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Identification of *acoR*, a Regulatory Gene for the Expression of Genes Essential for Acetoin Catabolism in *Alcaligenes eutrophus* H16

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Two hundred thirty-nine base pairs upstream from *acoXABC*, which encodes the *Alcaligenes eutrophus* H16 structural genes essential for cleavage of acetoin, the 2,004-bp *acoR* gene was identified. *acoR* encodes a protein of 668 amino acids with a molecular mass of 72.9 kDa. The amino acid sequence deduced from *acoR* exhibited homologies to the primary structures of transcriptional activators such as NifA of *Azotobacter vinelandii*, NtrC of *Klebsiella pneumonie*, and HoxA of *A. eutrophus*. Striking similarities to the central domain of these proteins and the presence of a typical nucleotide-binding site (GETGSGK) as well as of a C-terminal helix-turn-helix motif as a DNA-binding site were revealed. Between *acoR* and *acoXABC*, two different types of sequences with dual rotational symmetry [CAC-(N_{11} to N_{18})-GTG and TGT-(N_{10} to N_{14})-ACA] were found; these sequences are similar to NtrC and NifA upstream activator sequences, respectively. Determination of the N-terminal amino acid sequence of an *acoR'-'lacZ* gene fusion identified the translational start of *acoR*. S1 nuclease protection assay identified the transcriptional start site 109 bp upstream of *acoR*. The promoter region (TTGCGC-N_{18}-TACATT) resembled the σ^{70} consensus sequence of *Escherichia coli*. Analysis of an *acoR'-'lacZ* fusion and primer extension studies revealed that *acoR* was expressed at a low level under all culture conditions, whereas *acoXABC* was expressed only in acetoin-grown cells. The insertions of Tn*5* in six transposon-induced acetoin-negative mutants of *A. eutrophus* were mapped within *acoR*. On the basis of these studies, it is probable that AcoR represents a regulatory protein which is required for σ^{54}-dependent transcription of *acoXABC*.

The aerobic bacterium *Alcaligenes eutrophus* cleaves acetoin oxidatively into two C_2 compounds. Whereas one compound is acetaldehyde, which is further oxidized to acetate, the second compound has been identified as acetate by in vitro experiments. However, it is assumed that acetyl coenzyme A is produced in vivo as the second product of the cleavage reaction, as in the anaerobic bacterium *Pelobacter carbinolicus* (33). During cultivation on acetoin, *A. eutrophus* induces the formation of an acetoin:2,6-dichlorophenolindophenol oxidoreductase (Ao:DCPIP OR) (17, 49), which is composed of two different subunits and whose primary structures exhibit homologies to the α and β subunits of the E2 components of 2-oxo acid dehydrogenase complexes (34). In addition to Ao:DCPIP OR, a fast-migrating protein (FMP), which is most probably a dihydrolipoamide acetyltransferase (34), and acetaldehyde dehydrogenase II (AcDH-II), which exhibits a high affinity towards acetaldehyde (23), are induced under these conditions (34, 49).

Together with *acoX*, which encodes a protein of yet unknown function, the structural genes for both subunits of Ao:DCPIP OR (*acoA* and *acoB*) and for FMP (*acoC*) are organized as *acoXABC* in one operon (34). The transcription of the *aco* operon (17, 34) and of *acoD*, which is the structural gene for AcDH-II (35), as well as of some other genes of *A. eutrophus* (39, 56), depends on the presence of a sigma factor encoded by an *ntrA*-like gene. *acoXABC*, *acoD*, and *hoxFUYH* are preceded by regions which resemble the σ^{54}-dependent promoter consensus sequence of enterobacteria, and the transcriptional start sites have been identified downstream from these regions (34, 35, 56).

The subject of this study was the region upstream of the *acoXABC* operon which is characterized by insertional inactivation by Tn*5* in some transposon-induced acetoin-negative mutants. We have identified and analyzed at a molecular level the structural gene (*acoR*) for a protein which is essential for the expression of proteins involved in the conversion of acetoin to intermediates of the central metabolism. This study indicates that a positive activator protein is involved in the expression of these genes and that the regulation may follow the common pattern of regulation of genes which are preceded by σ^{54}-dependent promoters (25).

MATERIALS AND METHODS

Bacterial strains and plasmids. The strains of *A. eutrophus* and *Escherichia coli* and the plasmids used in this study are listed in Table 1.

Growth of bacteria. *E. coli* was grown at 37°C in Luria-Bertani medium or in M9 medium (41) containing 0.2% (wt/vol) glucose. *A. eutrophus* was grown at 30°C either in a complex medium of nutrient broth (0.8%, wt/vol) or in a mineral salts medium (43).

Isolation of RNA and DNA. Total RNA was isolated as described by Oelmüller et al. (32). Plasmid DNA was prepared from crude lysates by the alkaline extraction procedure (8, 41).

Analysis and manipulation of DNA. Isolated plasmid DNA was digested with various restriction endonucleases under the conditions described by Sambrook et al. (41) or by the manufacturer. DNA restriction fragments were isolated from

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TABLE 1. Bacterial strains, plasmids, bacteriophage, and DNA fragments used in this study

Strain, plasmid, phage, or fragment	Relevant characteristics	Source or reference
Alcaligenes eutrophus H16	Wild type, autotrophic, prototrophic	DSM 428, ATCC 17699 (60)
Escherichia coli		
DH1	*recA1 thi-1*	19
S17-1	*recA*, harbors the *tra* genes of plasmid RP4 in the chromosome, *proA thi-1*	47
XL-1 Blue	*recA1 endA1 gyrA96 thi hsdR17* (r_K^- m_K^+) *supE44 relA1* λ^- *lac* [F' *proAB lacI*^{q}ZΔM15, Tn*10*(Tet)]	10
Phage M13K07	Km^r	Pharmacia-LKB
Plasmids		
pUC9-1	Ap^r *lacPOZ'*	20
pMC1403	Ap^r *lacZ'* Tc^r	11
pMP92	Tc^r	50
pBluescript SK$^-$	Ap^r *lacPOZ'*, T7 and T3 promoter	Stratagene
pRZ1	pMC1403 derivative, *acoR'-'lacZ*	This study
pRZ10	pMP92 derivative, *acoR'-'lacZ*	This study
DNA fragments[a]		
A	Genomic *Eco*RI restriction fragment of *A. eutrophus* harboring genes essential for acetoin catabolism	17
AT^{H1098}, AT^{N1230}, AT^{N1236}, AT^{N1240}, AT^{N1244}, AT^{N1246}, and AT^{N1247}	Tn*5*-harboring derivatives of fragment A from acetoin catabolism mutants of *A. eutrophus*	17
EE35	*acoR* harboring subfragment of A	34

[a] Superscripts refer to acetoin catabolism mutants of *A. eutrophus*.

agarose gels by using a Geneclean kit (58) or by electroelution into a sodium acetate solution in an apparatus obtained from Biometra (Göttingen, Germany). Restricted DNA was ligated to vectors which had been dephosphorylated by treatment with calf intestinal phosphatase prior to ligation. Recessed 3' ends of restricted DNA molecules were filled in with the Klenow enzyme as described by the manufacturer, Bethesda Research Laboratories (Gaithersburg, Md.) (6a).

Transfer of DNA. For transformation, *E. coli* was grown aerobically in Luria-Bertani medium containing 20 mM $MgCl_2$ at 37°C. Competent cells were prepared and transformed by the calcium chloride procedure described by Sambrook et al. (41). Mating of *A. eutrophus* (recipient) with *E. coli* S17-1 (donor) harboring hybrid donor plasmids was performed on solidified nutrient broth medium as described by Friedrich et al. (16).

Synthesis of oligonucleotides. Oligonucleotides were synthesized in 0.2-μmol portions from deoxynucleoside phosphoamidites (4) in a Gene Assembler Plus apparatus according to the protocol described by the manufacturer (Pharmacia-LKB, Uppsala, Sweden). The oligonucleotides were released from the support matrix, and the protection groups were removed by 15 h of incubation at 55°C in 25% (vol/vol) ammonium. The oligonucleotides were finally purified by passage through a NAP-10 column (Pharmacia-LKB).

DNA sequence analysis. DNA sequencing was carried out by the dideoxy-chain termination method of Sanger et al. (42) with single-stranded or double-stranded alkali-denatured plasmid DNA, *d*7-deaza-GTP instead of dGTP (30), and [α-^{35}S]dATP by using a T7 polymerase sequencing kit according to the manufacturer's protocol (Pharmacia-LKB). Single strands were prepared with the helper phage M13K07. Synthetic oligonucleotides were used as primers, and the "primer-hopping strategy" (51) was employed. The products of the sequencing reactions were separated in 8% (wt/vol) acrylamide gels in buffer (pH 8.3) containing 100 mM hydrochloride, 83 mM boric acid, 1 mM EDTA, and 42% (wt/vol) urea in an S2 sequencing apparatus (GIBCO/BRL Bethesda Research Laboratories GmbH, Eggenstein, Germany) and were visualized on X-ray films.

Analysis of sequence data. Nucleic acid sequence data and deduced amino acid sequences were analyzed with the Sequence Analysis Software Package (version 6.2, June 1990) by the method of Devereux et al. (13).

Determination of the transcriptional start site. To determine the transcriptional start site, primer extension as described by Sambrook et al. (41) and nuclease protection assays were used. The hybridization conditions for the S1 nuclease protection assays were as described in detail by Berk and Sharp (6) and Sambrook et al. (41), and the S1 nuclease reactions were conducted by the method described by Aldea et al. (1). DNA probes and dideoxynucleotide sequencing reactions for sizing the signals were performed with pBluescript SK$^-$::EE35 DNA as a template. In the annealing reaction, an oligonucleotide (5'-CTCGATGTGC TCGCGTT-3') complementary to positions 464 to 480 was used for ^{32}P labeling. For all mapping experiments, 25 μg of RNA was mixed with the labeled DNA fragments; the specific labeling rate was higher than 10^7 cpm/μg of DNA.

Preparation of crude extracts. Approximately 50 g (wet weight) of cells was suspended in 100 ml of buffer A (50 mM Tris hydrochloride [pH 7.4], 0.8% [vol/vol] Triton X-100, 10 mM magnesium chloride, 10 mM EDTA) supplemented with 200 μg of phenylmethylsulfonyl fluoride per ml. The cells were sonicated in a TG 250 sonicator (Schoeller & Co., Frankfurt, Germany).

Isolation of *lacZ* fusion proteins. All steps were carried out at 4°C. After phenylmethylsulfonyl fluoride was added to a final concentration of 2 mM to the crude extract obtained from *A. eutrophus* H16 harboring pRZ10, cellular debris was

removed by 20 min of centrifugation at 13,000 × *g* in an OTD50B centrifuge (Du Pont de Nemours GmbH, Bad Homburg, Germany). The supernatant (160 ml) was applied onto a DEAE-Sephacel column (5 by 10 cm; 195-ml bed volume), and the column was washed with 2 bed volumes of buffer A. The fusion protein was eluted with a linear NaCl gradient (150 to 600 mM) in buffer A. Fractions containing high levels of enzyme activity were combined, concentrated, washed with TBST buffer (50 mM Tris-HCl [pH 7.3], 150 mM NaCl, 0.2% [vol/vol] Triton X-100) by ultrafiltration in a Diaflo chamber with a YM5 membrane, and then applied to a ProtoSorb *lacZ* immunoaffinity adsorbent column (Promega Corporation, Madison, Wis.) which had been equilibrated with TBST buffer. The column was washed with 300 ml of TBST buffer, and proteins were eluted with 100 mM $NaHCO_3$-Na_2CO_3 (pH 10.8). Collected fractions were combined, concentrated and desalted by ultrafiltration using a Diaflo chamber and Microsep OD000C40 (Filtron Technology Corporation, Karlstein, Germany).

Determination of β-galactosidase activity. The activity of β-galactosidase was determined by hydrolysis of *o*-nitrophenol-β-D-galactopyranoside according to the method of Miller (28). The protein concentration was estimated by the method described by Lowry et al. (27) or the method described by Schmidt et al. (45).

Electrophoretic methods. Sodium dodecyl sulfate (SDS)- and mercaptoethanol-denatured proteins were separated in 8% (wt/vol) polyacrylamide gels in Tris-glycine buffer (25 mM Tris, 190 mM glycine, 0.1% [wt/vol] SDS) (26). Proteins were stained with Coomassie brilliant blue (59) or by the silver stain procedure (31, 52).

N-terminal sequence analysis. The sequence analysis was performed with a 477A pulsed liquid-phase protein-peptide sequencer (21) and a 120A on-line phenylthiohydantoin amino acid analyzer (38) according to the instructions of the manufacturer (Applied Biosystems, Weiterstadt, Germany).

Chemicals. Monoclonal anti-β-galactosidase mouse immunoglobulin G's and a ProtoSorb *lacZ* immunoaffinity adsorbent column were obtained from Promega. Restriction endonucleases, a nick translation kit, a DNA detection kit, RNA molecular weight markers, T4 DNA ligase, and DNA-modifying enzymes were obtained from GIBCO/BRL Bethesda Research Laboratories. RNase-free DNase, agarose NA, Sephadex G-50 (DNA grade), and phosphoamidites were from Pharmacia-LKB. Formaldehyde was from Sigma Chemical Co. (Gauting, Germany). Formamide, ethidium bromide, and EDTA were from Serva Feinbiochemica GmbH & Co. (Heidelberg, Germany). Complex media were from Difco Laboratories (Detroit, Mich.). All other chemicals were of the highest purity available from E. Merck AG (Darmstadt, Germany).

Nucleotide sequence accession number. The nucleotide and amino acid sequence data reported here have been submitted to the EMBL, GenBank, and DDBJ nucleotide sequence data bases under accession number M90471.

RESULTS

Determination of the gene locus and nucleotide sequence of *acoR*. The insertions of Tn*5* in mutants of *A. eutrophus* impaired in acetoin catabolism have been mapped on five different *Eco*RI fragments (A, B, C, D, and E) which were cloned recently (17). In six mutants, Tn*5* was mapped on fragment A and upstream of the *acoXABC* operon; these mutants did not express the proteins encoded by *acoXABC* or *acoD* (17, 34, 35). Therefore, a 3.5-kbp *Eco*RV-*Eco*RI subfragment of A, which is referred to as EE35 (Fig. 1) (34) and which included the region of Tn*5* insertion sites in the six mutants mentioned above, was cloned to the Bluescript vectors SK^- and KS^-. By using universal primers and synthetic oligonucleotides as primers, the nucleotide sequence of this fragment was determined from both strands by the dideoxy-chain termination method and the primer-hopping strategy (Fig. 2). An open reading frame (ORF) designated ORF1, which started at the ATG at position 244 and extended to position 2454 (Fig. 2), was identified. A second ORF, which started at position 2692 (Fig. 2), represented the 5′ region of *acoX* (34). Several other ORFs were detected (Fig. 1). However, none of them obeyed the rules of Bibb et al. (7) for a coding region or was preceded by a reliable ribosome-binding site.

Identification of the translation start. Three ATG codons at positions 244, 451, and 607 could represent the putative translational start codon of the new locus which was referred to as *acoR*. To determine the translational start site, a fusion gene which consisted of the 5′ region of *acoR* and of the *E. coli lacZ′* gene was constructed. The 0.8-kbp *Eco*RV-*Sal*I subfragment ES08, which was obtained from EE35 (Fig. 1), was treated with *Bal* 31, and the deleted fragments were ligated to the *Sma*I site of promoter probe vector pMC1403. Clones of *E. coli* XL-1 Blue, which formed blue colonies on Luria-Bertani X-Gal (5-bromo-4-chloro-3-indolyl-β-D-galactopyranoside) agar plates, were isolated. From one hybrid plasmid, which was referred to as pRZ1, a 6.6-kbp *Eco*RI-*Sal*I subfragment harboring the *acoR′-′lacZ* fusion was isolated and ligated to *Eco*RI- and *Xho*I-treated DNA of broad-host-range vector pMP92 (48) (Fig. 3). The resulting plasmid, pRZ10, was transferred to *A. eutrophus* H16 by conjugation. It harbored the region extending from position 10 to 759 of the sequence shown in Fig. 2 and included all potential ATG start codons.

The AcoR′-′LacZ fusion protein was purified from crude extracts obtained from acetoin-grown cells of the transconjugant which were harvested in the exponential growth phase. Subsequent anion-exchange chromatography on DEAE-Sephacel and affinity chromatography on *p*-aminophenyl-β-D-thiogalactopyranoside (APTG)–Sepharose yielded insufficient amounts of the fusion protein, probably because the binding of AcoR′-′LacZ to APTG-Sepharose was weak. The preparation of the AcoR′-′LacZ fusion protein was subsequently applied onto a ProtoSorb *lacZ* immunoaffinity adsorbent column, and this step yielded a protein with an M_r of 127,000 which appeared almost homogeneous as revealed by electrophoresis in SDS-polyacrylamide gels (Fig. 4). From the SDS-polyacrylamide gel, the proteins were transferred to a polyvinylidene difluoride membrane; that region of the membrane which harbored the AcoR′-′LacZ fusion protein was cut off and subjected to N-terminal amino acid sequence analysis. The obtained sequence (M D L R Q R E H I E) was consistent with translation initiation from the ATG start codon at position 451. In addition, only this ATG was preceded by a reliable Shine-Dalgarno sequence (Fig. 2) (see reference 50 for an overview). Therefore, *acoR* encoded a polypeptide of 668 amino acids with a predicted molecular mass of 72.9 kDa (Fig. 1).

Structure of the gene product. The G+C content of 71.3 mol% for *acoR* was slightly higher than the value of 66.3 to 66.9 mol% determined for total genomic DNA of *A. eutrophus* (12). Because of this high G+C content, a strong biased choice occurred for codons with either G or C in position 3 and position 1. The G+C contents amounted to 75.0, 47.9,

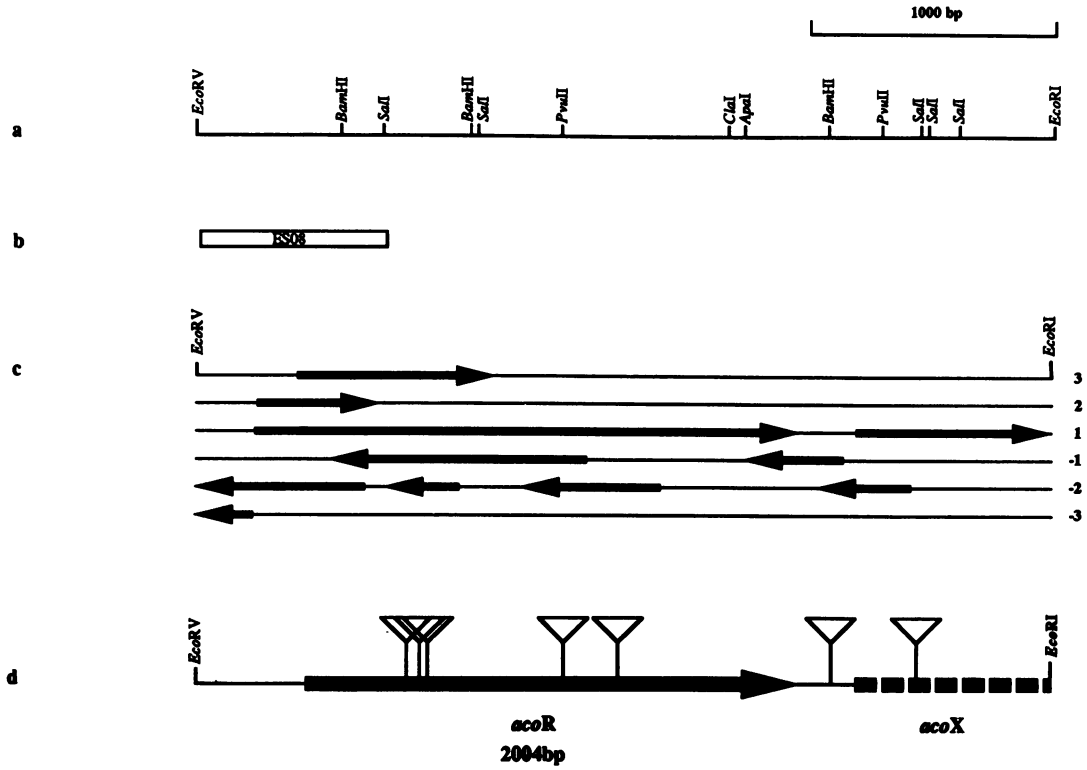

FIG. 1. Molecular organization of the *A. eutrophus acoR* gene locus. (a) Physical map of the region analyzed in this study. (b) Subfragment relevant for construction of the *acoR'-'lacZ* fusion. (c) Positions and orientations of ORFs comprising more than 150 nucleotides identified in this study (numbers indicate all six possible frames). (d) Orientation of *acoR* and position relative to *acoX*. Locations of transposon insertions determined by sequence analysis of genomic DNA fragments obtained from the mutants are indicated by triangles.

and 90.1 mol% for codon positions 1, 2, and 3, respectively, and they were close to the theoretical values of 71.5, 48.0, and 93.0 mol% which were calculated by the method of Bibb et al. (7). The codon usage corresponded mostly to those obtained for the gene products of *adh* (24), *rbcL* (2), and the *acoXABC* operon (33) and many other genes of *A. eutrophus*. However, AcoR exhibited a much higher content of Arg residues (9.5 mol%) and a lower content of Lys residues (0.9 mol%) than the products of the genes mentioned above or most other prokaryotic proteins (37, 57). A high Arg/Lys ratio is a feature of transcriptional activators belonging to the LysR family. The Arg residues in these DNA-binding proteins may constitute positively charged binding sites interacting with the phosphate moieties of DNA (37, 57).

Sequence alignments of the amino acid sequence deduced from *acoR* exhibited homologies to the regulatory proteins NtrC and NifA from various bacteria (Fig. 5 and 6). The overall amino acid identities were 31.8% with NifA of *Azotobacter vinelandii* (5), 35.5 and 32.7% with NtrC of *Rhizobium meliloti* (53) and *Klebsiella pneumoniae* (3), respectively, and 35.9% with GlnG of *E. coli* (29). The highly conserved central domains of these activator proteins, which are responsible for the interaction of the protein with the σ^{54}-dependent RNA polymerases (54), exhibited an even higher homology to the central region of AcoR, and the fraction of identical amino acids varied between 37 and 48%. The domain also included the conserved region, which represents the putative ATP-binding site (Fig. 5). The C-terminal region contains a high fraction of nonpolar residues and a helix-turn-helix motif, which is characteristic of DNA-binding proteins (Fig. 5). In contrast, the N-terminal region of *acoR* exhibited no significant homologies to the corresponding region of NtrC, NifA, or XylR or to any other gene product whose sequence is available from the EMBL data bank.

In the region ranging from 144 to 115 nucleotides upstream of *acoR*, we identified a sequence (TTGCGC-N_{18}-TACATT) which exhibited strong homology to the *E. coli* σ^{70} consensus promoter (TTGACA-N_{17}-TATAAT) (40). Upstream of *acoR* and in the intergenic region upstream of *acoX* at positions 267 to 281 and 2470 to 2493, respectively, regions of dual rotational symmetry were located (Fig. 2); both resembled the NtrC-binding site as proposed by Gussin et al. (18). Downstream of the latter motif, a repeating sequence was identified at positions 2503 to 2542; this sequence was identical with the consensus of upstream activator sequences from NifA (9).

Mapping of Tn*5* insertions. The insertions of Tn*5*::*mob* in the acetoin-negative mutants mentioned above had already been mapped roughly on fragment A (17). In this study we cloned the *Sal*I restriction fragments AT^{N1230}, AT^{N1236}, AT^{N1244}, AT^{N1246}, AT^{N1247}, and AT^{H1098}, which harbored genomic DNA of the respective mutant (17; Table 1) and of the adjacent Tn*5* DNA including the kanamycin resistance gene, into pUC9-1 DNA. Nucleotide sequencing identified the exact positions of Tn*5* in the mutant fragments by primer hybridization at a distance of 63 to 79 bp from the end of IS*50*L as described in detail by Priefert et al. (34) for *acoXABC*. In each of the mutant strains examined in this

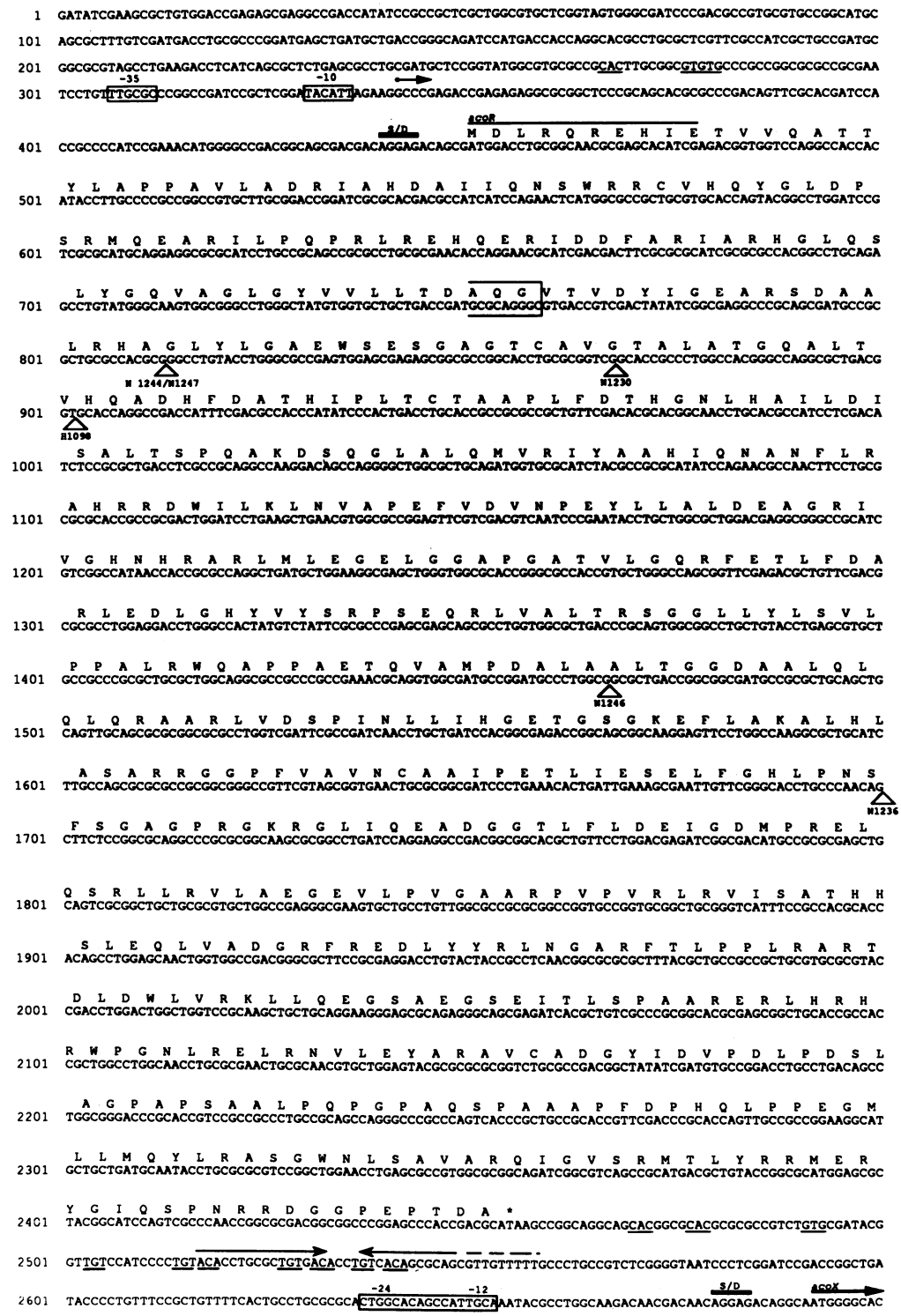

FIG. 2. Nucleotide sequence of the *A. eutrophus acoR* gene locus. Amino acids deduced from the nucleotide sequence are specified by standard one-letter abbreviations. The amino acid sequence determined for the N terminus of the AcoR'-'LacZ fusion is overlined. The 3' region of the AcoR' portion of the fusion is indicated by a bracket. A putative ribosome-binding site is indicated by a black bar and S/D. The position of a tentative factor-independent transcriptional terminator downstream of *acoR* is indicated by inverted arrows. The positions of Tn*5* insertions in acetoin-negative mutants are indicated by triangles with the designations of the mutants. Putative NifA- and NtrC-like upstream activator sequences are underlined. A dot and arrow indicate the transcription start site and the direction of transcription, as determined by S1 nuclease protection assay. The thick arrow labeled *acoX* indicates the 5' terminus of *acoX*.

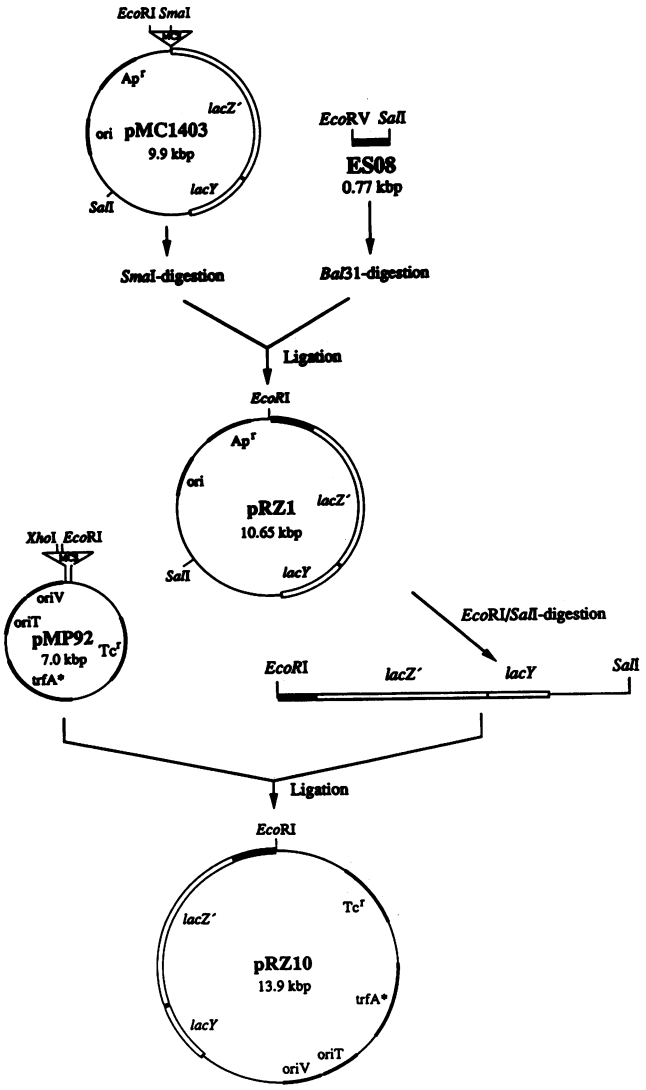

FIG. 3. Construction of plasmids for the identification of the translation start site. Abbreviations: MCS, multiple-cloning site; Ap^r, ampicillin resistance determinant; Tc^r, tetracycline resistance determinant; trfA*, a gene segment encoding the *trfA* Δ*kilD* replication protein (44); oriV, RK2 700-bp *oriV* region; oriT, RK2 760-bp *oriT* region (44); ori, ori of pMC1403 (11).

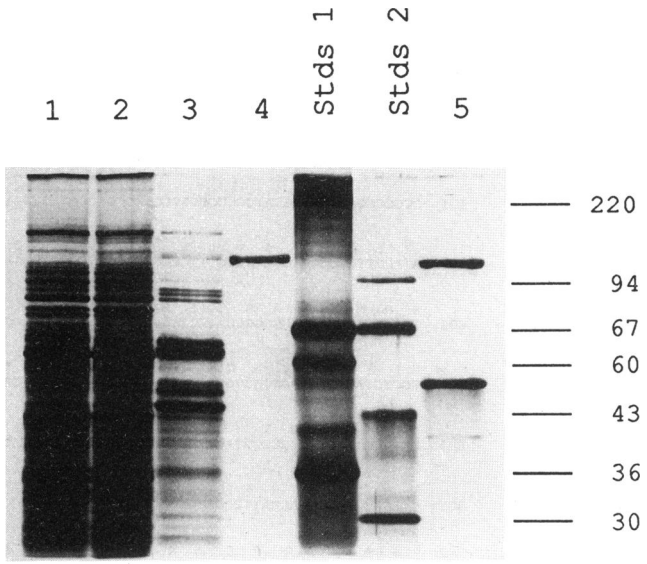

FIG. 4. Purification of the AcoR'-'LacZ fusion protein from acetoin-grown cells of *A. eutrophus* H16(pRZ10). Samples were applied onto SDS–8% (wt/vol) polyacrylamide gels, separated, and silver stained, as described in Materials and Methods. Lanes: 1, 20 μg of protein from crude extract of *A. eutrophus* H16; 2, 20 μg of protein from crude extract of *A. eutrophus* H16(pRZ10); 3, 5 μg of protein obtained from chromatography of *A. eutrophus* H16(pRZ10) crude extract on DEAE-Sephacel; 4, 1.5 μg of *A. eutrophus* H16(pRZ10) protein obtained from immunoaffinity chromatography on ProtoSorb *lacZ*; 5, crude extract of *E. coli* XL-1 Blue(pBluescript SK$^-$) expressing β-galactosidase (116 kDa) enriched by APTG chromatography; Stds 1 and Stds 2, standard proteins (molecular masses indicated on the right in kilodaltons).

study, the insertion of Tn*5*::*mob* was localized within *acoR* (Fig. 1 and 2).

Identification of the promoter and the putative transcriptional termination site. To prove the significance of the putative promoter structure (Fig. 2), S1 nuclease protection and primer extention assays (data not shown) with total RNA isolated from acetoin-grown cells of *A. eutrophus* strain H16 harvested in the exponential growth phase were performed. The transcriptional start site was identified 6 nucleotides downstream of the putative promoter consensus sequence at position 342 (Fig. 2 and 7).

An inverted repeat was located 60 bp downstream from the translational termination codon of *acoR* (Fig. 2). It may represent a factor-independent transcriptional terminator, and the free energy of this structure, calculated by the method of Tinoco et al. (55), is approximately −86.7 kJ/mol.

Determination of the promoter strength and regulation of *acoR* expression. The hybrid plasmid pRZ10, which contained the promoter region and the 5′ region of *acoR* fused to the promoterless *lacZ'* gene, was used to study the expression of *acoR*. With *A. eutrophus* H16 harboring pRZ10, β-galactosidase activity was detected at almost the same level at the exponential growth phase irrespective of the carbon source provided to the cells for growth. Entering the stationary growth phase, the β-galactosidase activity decreased to approximately 30% of the value obtained for the exponential phase (Table 2). The β-galactosidase activity conferred by the *acoR'-'lacZ* fusion was much lower (9.8 to 37.4 U/mg of protein [Table 2]) than that conferred by the *phbC'-'lacZ* fusion (534 U/mg of protein [46]) or by the *phbH'-'lacZ* fusion (482 U/mg of protein [36]) of *A. eutrophus* H16. It is not known whether these fusion proteins were different with respect to their stability or whether the translation efficiencies were different. However, these results correlated with the results obtained with the S1 protection assays shown in Fig. 7. The transcriptional start site of *acoR* was detectable with total RNA preparations from acetoin- or gluconate-grown cells of *A. eutrophus* H16 (Fig. 7, lanes 1 and 2) or from gluconate-grown cells of the mutant strain H1098 (Fig. 7, lane 3) which harbored Tn*5* in *acoR*. The stronger signals in lanes 4 and 5 probably result from an increased number of transcripts due to the presence of pRZ10. In contrast to the *acoR* promoter, the σ^{54}-dependent promoter of *acoXABC* was active only in acetoin-grown cells, as revealed by primer extension (data not shown) and by an S1 nuclease protection assay in an earlier study (35).

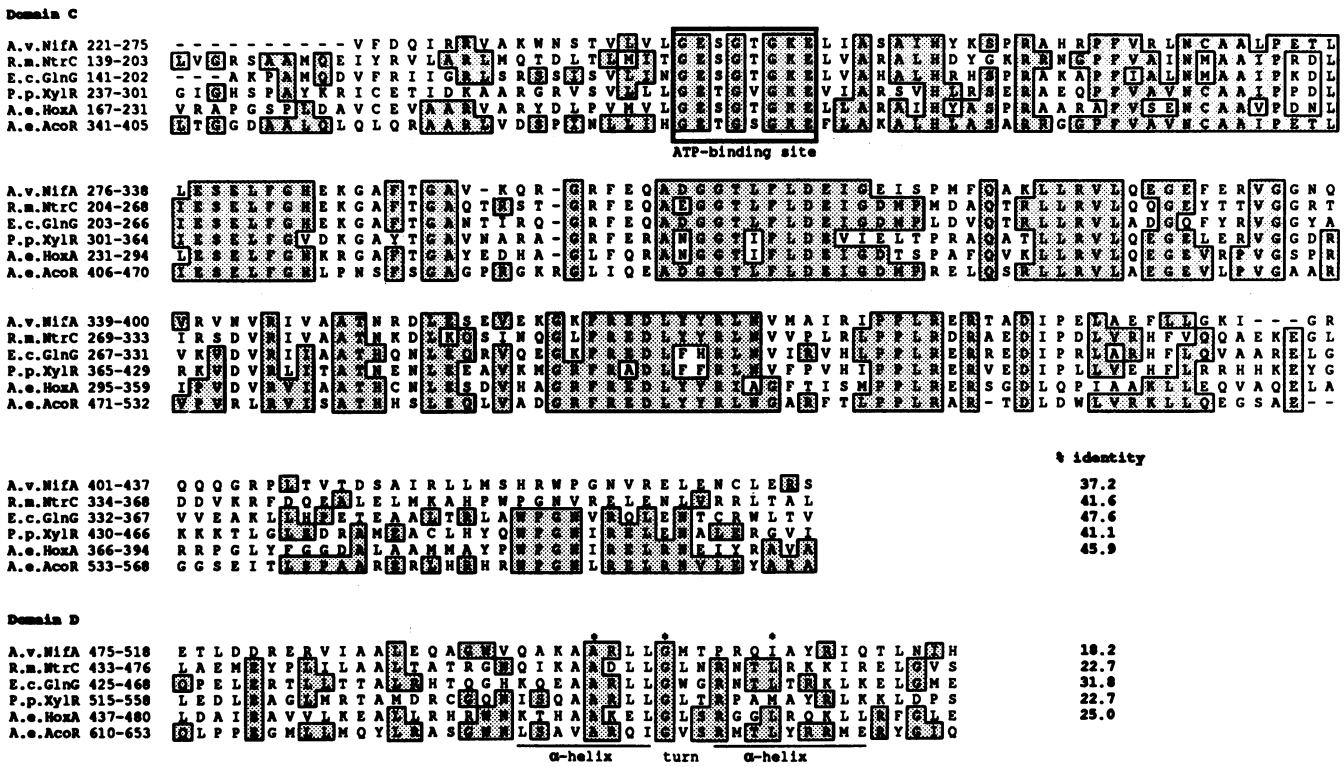

FIG. 5. Homology of the putative *acoR* gene product to other regulatory proteins. Amino acids of domains C and D of NifA (*A. vinelandii*) (5), NtrC (*R. meliloti*) (53), GlnG (*E. coli*) (29), XylR (*P. putida*) (22), HoxA (*A. eutrophus*) (15), and AcoR (*A. eutrophus*) are aligned. Matching amino acids are shaded and boxed. Dashed lines indicate gaps which were introduced to optimize the alignment. A putative ATP-binding site and a helix-turn-helix motif are indicated. Asterisks mark conserved amino acids in the helix-turn-helix motif. Numbers indicate the positions of the amino acids in the respective proteins. The degree of homology (percent identical amino acids) of each protein to the putative *acoR* gene product is indicated.

DISCUSSION

In the present study, we identified and sequenced *acoR*, a gene locus required for the expression of genes essential for acetoin catabolism in *A. eutrophus*, such as *acoXABC*. The data obtained from amino acid sequence comparison revealed strong evidence for a regulatory nature of AcoR and indicated that AcoR belongs to the NifA family of transcriptional activators. Homologies occurred with the central region of approximately 240 amino acids, including a puta-

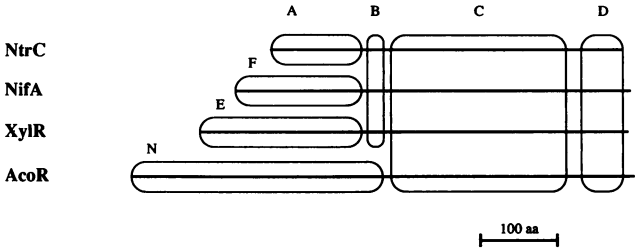

FIG. 6. Domain relationships in some characterized activating proteins of −24/−12 promoters (NtrC, NifA, XylR, and AcoR). The proteins are shown from the N termini (left) to the C termini (right) in a relative length corresponding to the number of amino acids (aa). Homologous sequences are boxed and denoted B, C, and D according to the notation of Thöny and Hennecke (54). Domains A, E, F, and N in the proteins are assumed to determine specific functions. For NifA, only type I (as it exists in *K. pneumoniae* and *A. vinelandii*) is shown.

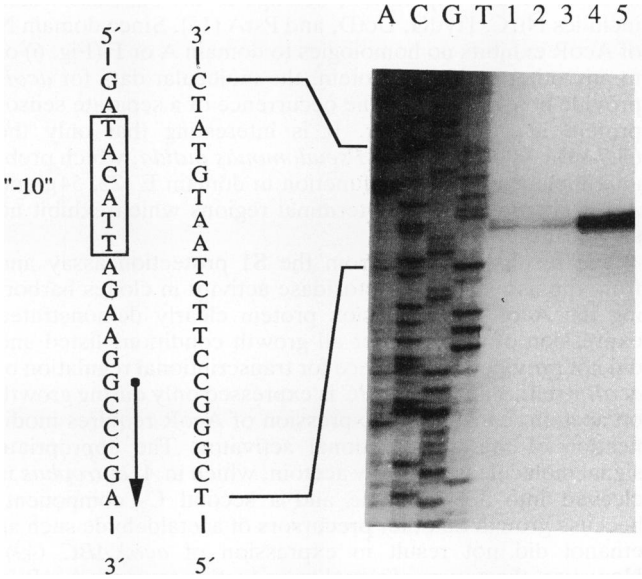

FIG. 7. S1 nuclease protection assays of the transcripts of *acoR* and *acoR'-'lacZ*. Lanes A, C, G, and T, standard sequencing reactions to size the mapping signals. RNA was isolated from acetoin-grown cells of *A. eutrophus* H16 (lane 1), gluconate-grown cells of *A. eutrophus* H16 (lane 2), gluconate-grown cells of *A. eutrophus* mutant H1098 (lane 3), acetoin-grown cells of *A. eutrophus* H16(pRZ10) (lane 4), and gluconate-grown cells of *A. eutrophus* H16(pRZ10) (lane 5).

TABLE 2. β-Galactosidase activities of the AcoR'-'LacZ fusion protein in *A. eutrophus* H16 during growth on various carbon sources

Plasmid contained in H16	Substrate[a]	Sp act (U/mg)[b] in indicated growth phase	
		Exponential	Stationary
None	Acetoin	0.0	0.0
pRZ10[c]	Acetoin	37.4	12.3
	Gluconate	32.9	9.8
	Fructose	29.8	12.9
	Succinate	36.8	15.4

[a] The substrates were added from filter-sterilized stock solutions at the following final concentrations: 0.3% (wt/vol) acetoin and 0.5% (wt/vol) gluconate, fructose, and succinate.
[b] Mean value of three cultures.
[c] The medium of *A. eutrophus* harboring pRZ10 was supplemented with tetracycline. The structure of pRZ10 is shown in Fig. 3.

tive ATP-binding site, and with the C-terminal DNA-binding domain (54). When the putative helix-turn-helix motif was scored for similarities to helix-turn-helix motifs of known DNA-binding domains by the method of Dodd and Egan (14), a score of 5.95 was obtained for the 22 amino acids at positions 627 to 648, thus confirming evidence for the presence of such a motif in AcoR (Fig. 6). The central and carboxy-terminal regions of these regulatory proteins are conserved, whereas the N-terminal regions, which provide specific regulatory functions, are often variable. Only those activators which are modified by sensor proteins interacting with the N termini of the regulators, such as NtrC and probably NifA (type I) (54), exhibit homologies of the N-terminal domains, e.g., domains A and F for NtrC and NifA (type I), respectively (Fig. 6). For example, the activator HoxA of *A. eutrophus*, which is involved in the control of hydrogenase expression, belongs to the subclass which includes NtrC, HydG, DctD, and PstA (15). Since domain N of AcoR exhibits no homologies to domain A or F (Fig. 6) or to any other activator protein, the molecular data for *acoR* provide no evidence for the occurrence of a separate sensor protein in *A. eutrophus*. It is interesting that only the 63.7-kDa XylR protein of *Pseudomonas putida*, which probably includes the sensor function in domain E (22, 54), and AcoR share extended N-terminal regions which exhibit no significant homology.

The results obtained from the S1 protection assay and from the assay of β-galactosidase activity in clones harboring the AcoR'-'LacZ fusion protein clearly demonstrated expression of AcoR under all growth conditions listed and did not provide any evidence for transcriptional regulation of *acoR* itself. Since *acoXABC* is expressed only during growth on acetoin, constitutive expression of AcoR requires modification of the transcriptional activator. The appropriate signal molecule is probably acetoin, which in *A. eutrophus* is cleaved into acetaldehyde and a second C_2 component, because growth on other precursors of acetaldehyde such as ethanol did not result in expression of *acoXABC* (49). However, the nature of signal transduction towards AcoR in *A. eutrophus* remains unclear.

Approximately 100 to 200 bp upstream of many −24/−12 promoters, conserved enhancer sequences which bind activator proteins for maximum expression have been identified (54). The TGT-N_{10}-ACA motif, which was identified 95 to 110 bp upstream of the promoter of *acoXABC*, is identical to the consensus of 19 *nif* promoters of various species (18). In addition, a CAC-N_{11}-GTG motif 144 to 160 bp upstream of the *acoXABC* promoter, which resembled the NtrC upstream activator sequence, was detected (9). Both regions may represent upstream activator sequences which bind to the DNA binding domain of AcoR or a modified, activated derivative of AcoR. The significance of an additional potential activator target site upstream of the *acoR* promoter, e.g., for autoregulation of *acoR* expression, remains to be elucidated.

Future studies will focus on the isolation and characterization of native AcoR protein in order to examine the interactions of AcoR with the putative binding sites upstream of *acoXABC* and *acoR* and to understand the mechanism of transcriptional activation. The identification of the signal molecule and understanding of the regulatory cascade will contribute to the understanding of positively controlled gene expression in *A. eutrophus*. These studies will also reveal whether the expression of other genes which are essential for acetoin catabolism in *A. eutrophus* also depends on the presence of AcoR or whether additional regulator proteins are necessary for transcriptional activation of these genes, as they are for the expression of *acoD* (35).

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REFERENCES

1. **Aldea, M., F. Claverie-Martin, M. R. Diaz-Torres, and S. R. Kushner.** 1988. Transcript mapping using [^{35}S] DNA probes, trichloroacetate solvent and dideoxy sequencing ladders: a rapid method for identification of transcriptional start points. Gene **65**:101–110.
2. **Andersen, K., and J. Caton.** 1987. Sequence analysis of the *Alcaligenes eutrophus* chromosomally encoded ribulose bisphosphate carboxylase large and small subunit genes and their gene products. J. Bacteriol. **169**:4547–4558.
3. **Arnold, W., A. Rump, W. Klipp, U. B. Priefer, and A. Pühler.** 1988. Nucleotide sequence of a 24,206-base-pair DNA fragment carrying the entire nitrogen fixation gene cluster of *Klebsiella pneumoniae*. J. Mol. Biol. **203**:715–738.
4. **Beaucage, S. L., and M. H. Caruthers.** 1981. Deoxynucleoside phosphoamidites—a new class of key intermediates for deoxypolynucleotide synthesis. Tetrahedron Lett. **22**:1859–1862.
5. **Bennett, L. T., F. Cannon, and D. R. Dean.** 1988. Nucleotide sequence and mutagenesis of the *nifA* gene from *Azotobacter vinelandii*. Mol. Microbiol. **2**:315–321.
6. **Berk, A., and P. A. Sharp.** 1977. Sizing and mapping of early adenovirus mRNAs by gel electrophoresis of S1 endonuclease-digested hybrids. Cell **12**:721–732.

6a. **Bethesda Research Laboratories.** 1984. Methods to fill in 3' ends of DNA. Focus **6**:6.

7. **Bibb, M. J., P. R. Findlay, and M. W. Johnson.** 1984. The relationship between base composition and codon usage in bacterial genes and its use for the simple and reliable identification of protein-coding sequences. Gene **30**:157–166.
8. **Birnboim, H. C., and J. Doly.** 1979. A rapid alkaline extraction procedure for screening recombinant plasmid DNA. Nucleic Acids Res. **7**:1513–1523.
9. **Buck, M., S. Miller, M. Drummond, and R. Dixon.** 1986. Upstream activator sequences are present in the promoters of nitrogen fixation genes. Nature (London) **320**:374–378.
10. **Bullock, W. O., J. M. Fernandez, and J. M. Short.** 1987. XL1-Blue: a high efficiency plasmid transforming *recA Escherichia coli* strain with beta-galactosidase selection. BioTechniques **5**:376–379.
11. **Casadaban, M. J., J. Chou, and S. N. Cohen.** 1980. In vitro gene fusions that join an enzymatically active β-galactosidase seg-

ment to amino-terminal fragments of exogenous proteins: *Escherichia coli* plasmid vectors for the detection and cloning of translational initiation signals. J. Bacteriol. **143:**971–980.
12. **Davis, D. H., M. Doudoroff, and R. Y. Stanier.** 1969. Proposal to reject the genus *Hydrogenomonas*: taxonomic implications. Int. J. Syst. Bacteriol. **19:**375–390.
13. **Devereux, J., P. Haeberli, and O. Smithies.** 1984. A comprehensive set of sequence analysis programs for the VAX. Nucleic Acids Res. **12:**387–395.
14. **Dodd, I. B., and J. B. Egan.** 1990. Improved detection of helix-turn-helix DNA-binding motifs in protein sequences. Nucleic Acids Res. **18:**5019–5026.
15. **Eberz, G., and B. Friedrich.** 1991. Three *trans*-acting regulatory functions control hydrogenase synthesis in *Alcaligenes eutrophus*. J. Bacteriol. **173:**1845–1854.
16. **Friedrich, B., C. Hogrefe, and H. G. Schlegel.** 1981. Naturally occurring genetic transfer of hydrogen-oxidizing ability between strains of *Alcaligenes eutrophus*. J. Bacteriol. **147:**198–205.
17. **Fründ, C., H. Priefert, A. Steinbüchel, and H. G. Schlegel.** 1989. Biochemical and genetic analysis of acetoin catabolism in *Alcaligenes eutrophus*. J. Bacteriol. **171:**6539–6548.
18. **Gussin, G. N., C. W. Ronson, and F. M. Ausubel.** 1986. Regulation of nitrogen fixation genes. Annu. Rev. Genet. **20:** 567–591.
19. **Hanahan, D.** 1983. Studies on transformation of *Escherichia coli* with plasmids. J. Mol. Biol. **166:**557–580.
20. **Hanna, Z., C. Fregeau, G. Prefontaine, and R. Brousseau.** 1984. Construction of a family of universal expression plasmid vectors. Gene **30:**247–250.
21. **Hewick, R. M., M. W. Hunkapiller, L. E. Hood, and W. J. Dreyer.** 1981. A gas-liquid solid phase peptide and protein sequenator. J. Biol. Chem. **256:**7990–7997.
22. **Inouye, S., A. Nakazawa, and T. Nakazawa.** 1988. Nucleotide sequence of the regulatory gene *xlyR* of the TOL plasmid from *Pseudomonas putida*. Gene **66:**301–306.
23. **Jendrossek, D., A. Steinbüchel, and H. G. Schlegel.** 1987. Three different proteins exhibiting NAD-dependent acetaldehyde dehydrogenase activity from *Alcaligenes eutrophus*. Eur. J. Biochem. **167:**541–548.
24. **Jendrossek, D., A. Steinbüchel, and H. G. Schlegel.** 1988. Alcohol dehydrogenase gene from *Alcaligenes eutrophus*: subcloning, heterologous expression in *Escherichia coli*, sequencing, and location of Tn*5* insertions. J. Bacteriol. **170:**5248–5256.
25. **Kustu, S., E. Santero, J. Keener, D. Popham, and D. Weiss.** 1989. Expression of σ^{54} (*ntrA*)-dependent genes is probably united by a common mechanism. Microbiol. Rev. **53:**367–376.
26. **Laemmli, U. K.** 1970. Cleavage of structural proteins during the assembly of the head of bacteriophage T4. Nature (London) **227:**680–685.
27. **Lowry, O. H., N. J. Rosebrough, A. L. Farr, and R. J. Randall.** 1951. Protein measurement with the Folin phenol reagent. J. Biol. Chem. **193:**265–275.
28. **Miller, J. H.** 1972. Assay of β-galactosidase, p. 319, 353. *In* T. Platt, B. Miller-Hill, and J. H. Miller (ed.), experiments in molecular genetics. Cold Spring Harbor Laboratory, Cold Spring Harbor, N.Y.
29. **Miranda-Rios, J., R. Sanchez-Pescador, M. Urdea, and A. A. Covarrubias.** 1987. The complete nucleotide sequence of the *glnALG* operon of *Escherichia coli* K12. Nucleic Acids Res. **15:**2757–2770.
30. **Mizusawa, S., S. Nishimura, and F. Seela.** 1986. Improvement of the dideoxy chain termination method of DNA sequencing by use of deoxy-7-deazaguanosine triphosphate in place of dGTP. Nucleic Acids Res. **14:**1319–1324.
31. **Oakley, B. R., D. R. Kirsch, and N. R. Morris.** 1980. A simplified ultrasensitive silver stain for detecting proteins in polyacrylamide gels. Anal. Biochem. **105:**361–363.
32. **Oelmüller, U., N. Krüger, A. Steinbüchel, and C. G. Friedrich.** 1990. Isolation of prokaryotic RNA and detection of specific mRNA with biotinylated probes. J. Microbiol. Methods **11:**73–84.
33. **Oppermann, F. B., B. Schmidt, and A. Steinbüchel.** 1991. Purification and characterization of acetoin:2,6-dichlorophenolindophenol oxidoreductase, dihydrolipoamide dehydrogenase, and dihydrolipoamide acetyltransferase of the *Pelobacter carbinolicus* acetoin dehydrogenase enzyme system. J. Bacteriol. **173:**757–767.
34. **Priefert, H., S. Hein, N. Krüger, K. Zeh, B. Schmidt, and A. Steinbüchel.** 1991. Identification and molecular characterization of the *Alcaligenes eutrophus* H16 *aco* operon genes involved in acetoin catabolism. J. Bacteriol. **173:**4056–4071.
35. **Priefert, H., N. Krüger, D. Jendrossek, B. Schmidt, and A. Steinbüchel.** 1992. Identification and molecular characterization of the gene coding for acetaldehyde dehydrogenase II (*acoD*) of *Alcaligenes eutrophus*. J. Bacteriol. **174:**899–907.
36. **Pries, A.** Unpublished data.
37. **Riordan, J. F.** 1979. Arginyl residues and anion binding sites in proteins. Mol. Cell. Biochem. **26:**71–92.
38. **Rodriguez, H., W. J. Kohr, and R. N. Harkins.** 1984. Design and operation of a completely automated Beckman microsequencer. Anal. Biochem. **140:**538–547.
39. **Römermann, D., J. Warrelmann, R. A. Bender, and B. Friedrich.** 1989. An *rpoN*-like gene of *Alcaligenes eutrophus* and *Pseudomonas facilis* controls expression of diverse metabolic pathways, including hydrogen oxidation. J. Bacteriol. **171:**1093–1099.
40. **Rosenberg, M., and D. Court.** 1979. Regulatory sequences involved in the promotion and termination of RNA transcription. Annu. Rev. Genet. **13:**319–353.
41. **Sambrook, J., E. F. Fritsch, and T. Maniatis.** 1989. Molecular cloning: a laboratory manual, 2nd ed. Cold Spring Harbor Laboratory Press, Cold Spring Harbor, N.Y.
42. **Sanger, F., S. Nicklen, and A. R. Coulson.** 1977. DNA sequencing with chain-terminating inhibitors. Proc. Natl. Acad. Sci. USA **74:**5463–5467.
43. **Schlegel, H. G., H. Kaltwasser, and G. Gottschalk.** 1961. Ein Submersverfahren zur Kultur wasserstoffoxidierender Bakterien: Wachstumsphysiologische Untersuchungen. Arch. Mikrobiol. **38:**209–222.
44. **Schmidhauser, T. J., and D. R. Helinski.** 1985. Regions of broad-host-range plasmid RK2 involved in replication and stable maintenance in nine species of gram-negative bacteria. J. Bacteriol. **164:**446–455.
45. **Schmidt, K., S. L. Jensen, and H. G. Schlegel.** 1963. Die Carotinoide der Thiorhodaceae. I. Okenon als Hauptcarotinoid von *Chromatium okenii* Perty. Arch. Mikrobiol. **46:**117–126.
46. **Schubert, P.** 1990. Ph.D. thesis. Georg-August-Universität Göttingen, Göttingen, Germany.
47. **Simon, R., U. Priefer, and A. Pühler.** 1983. A broad host range mobilization system for *in vivo* genetic engineering: transposon mutagenesis in gram-negative bacteria. Bio/Technology **1:**784–791.
48. **Spaink, H. P., R. J. H. Okker, C. A. Wijffelman, E. Pees, and B. J. J. Lugtenberg.** 1987. Promoters in the nodulation region of the *Rhizobium leguminosarum* Sym plasmid pRL1JI. Plant Mol. Biol. **9:**27–39.
49. **Steinbüchel, A., C. Fründ, D. Jendrossek, and H. G. Schlegel.** 1987. Isolation of mutants of *Alcaligenes eutrophus* unable to derepress the fermentative alcohol dehydrogenase. Arch. Microbiol. **148:**178–186.
50. **Steinbüchel, A., N. Krüger, H. Valentin, A. Timm, A. Pries, E. Hustede, and H. G. Schlegel.** 1992. Physiological and genetic analysis of polyhydroxyalkanoate biosynthetic pathways, p. 315–327. *In* E. Galli, S. Silver, and B. Witholt (ed.), *Pseudomonas*: molecular biology and biotechnology. American Society for Microbiology, Washington, D.C.
51. **Strauss, E. C., J. A. Kobori, G. Siu, and L. E. Hood.** 1986. Specific-primer-directed DNA sequencing. Anal. Biochem. **154:** 353–360.
52. **Switzer, R. C., C. R. Merril, and S. Shifrin.** 1979. A highly sensitive silver stain for detecting proteins and peptides in polyacrylamide gels. Anal. Biochem. **98:**231–237.
53. **Szeto, W. W., B. T. Nixon, C. W. Ronson, and F. M. Ausubel.** 1987. Identification and characterization of the *Rhizobium meliloti ntrC* gene: *R. meliloti* has separate regulatory pathways

for activation of nitrogen fixation genes in free-living and symbiotic cells. J. Bacteriol. **169:**1423–1432.
54. **Thöny, B., and H. Hennecke.** 1989. The −24/−12 promoter comes of age. FEMS Microbiol. Rev. **63:**341–358.
55. **Tinoco, I., P. N. Borer, B. Dengler, M. D. Levine, O. C. Uhlenbeck, D. M. Crothers, and J. Gralla.** 1973. Improved estimation of secondary structure in ribonucleic acids. Nature (London) New Biol. **246:**40–41.
56. **Tran-Betcke, A., U. Warnecke, C. Böcker, C. Zaborosch, and B. Friedrich.** 1990. Cloning and nucleotide sequences of the genes for the subunits of NAD-reducing hydrogenase of *Alcaligenes eutrophus* H16. J. Bacteriol. **172:**2920–2929.
57. **Viale, A. M., H. Kobayashi, T. Akazawa, and S. Henikoff.** 1991. *rcbR*, a gene coding for a member of the LysR family of transcriptional regulators, is located upstream of the expressed set of ribulose 1,5-bisphosphate carboxylase/oxygenase genes in the photosynthetic bacterium *Chromatium vinosum*. J. Bacteriol. **173:**5224–5229.
58. **Vogelstein, B., and D. Gillespie.** 1979. Preparative and analytical purification of DNA from agarose. Proc. Natl. Acad. Sci. USA **76:**615–619.
59. **Weber, K., and M. Osborn.** 1969. The reliability of molecular weight determinations by dodecyl sulfate-polyacrylamide gel electrophoresis. J. Biol. Chem. **244:**4406–4412.
60. **Wilde, E.** 1962. Untersuchungen über Wachstum und Speicherstoffsynthese von *Hydrogenomonas*. Arch. Mikrobiol. **43:**109–137.